REMARKS

Status of the application

Claims 1-39, 40-42 and 44-45 were pending in the application, with claims 1-39 being withdrawn by the Examiner as directed to non-elected invention, and claims 40-42 and 44-45 stand rejected.

With entry of the instant response, claims 40, 48 and 54 have been amended to replace the recitation of "microbe" with "bacterium." Also, claims 40 and 54 have been amended to replace the recitation of "covalent attached to the antibody" with the phrase "conjugated to the antibody." Claim 40 has been further amended to specify that the source of singlet oxygen, in addition to being not conjugated to the antibody, is also not conjugated other molecules. Support for these amendments is implicitly and inherently present in the subject disclosure which taught mixing an antibody and a source of singlet oxygen that are not linked to each other or to other molecules and then assaying ozone production or bacterial killing. See the specification, e.g, at page 78, lines 1-4; page 83, lines 11-18; and page 93, lines 17-24.

Applicants provide the following remarks to address the various rejections raised in the instant Office Action.

Rejection under 35 U.S.C. §102

The instant office action maintained the rejection of claims 40-42, 48-51 and 54-56 as allegedly anticipated by Devanathan et al. (Proc. Nat'l. Acad. Sci. USA 87:2980-2984, 1990). In addition, claims 40-42, 44-45 and 48-58 were rejected as allegedly anticipated by Wentworth et al. (Proc. Natl. Acad. Sci. USA 97:10930-5, 2000) in light of Scripps Press Release (November 14, 2002). These rejections are addressed in turn below.

Applicants first note that, in maintaining the rejection over Devanathan et al. (Proc. Nat'l. Acad. Sci. USA 87:2980-2984, 1990), the Examiner alleged that the "wherein" clause recited in the claims does not carry any patentable weight. Applicants respectfully disagree. As explained in the MPEP, § 2111.04, a "wherein" clause is not limiting if it merely "suggests or makes optional but does not require steps to be performed, or by claim language that does not limit a claim to a particular structure" or if "simply expresses the intended result of a process step positively recited." This clearly does not apply to the present claims. Instead, the "wherein" clause in the present claims is material to patentability because it limits the structure of the components recited in the claims (i.e., the antibody and the source of singlet oxygen). It certainly carries patentable weight since, by further specifying what kind of materials to use, it materially affects the steps to be performed in the claimed methods.

Devanathan et al. does not anticipate the presently claimed invention because Devanathan discussed the use of a photodynamic dye that is conjugated to a goat anti-rabbit antibody in the selective killing of E. coli. In addition, the photodynamic dye needs to be exposed to light in order to be bactericidal. On the other hand, the present claims 40-45 specify that the recited source of singlet oxygen is not conjugated to the antibody or other molecules. As such, these claims are novel over Devanathan et al. Pending claims 48-58 are also novel over Devanathan et al. because these claims recite that the employed source of singlet oxygen would not kill the bacteria on its own <u>even when exposed to light</u>.

Turning to the rejection based on Wentworth et al., this reference also could not anticipate the subject invention because

it does not teach each and every element recited in the present claims. For example, the present claims specify generation of ozone to inhibit growth of a bacterium by using an antibody that can bind to the bacterium along with a source of singlet oxygen. There is not any teaching of ozone production in the cited art. In addition, while there is some discussion of antibody generated reactive oxygen species for killing bacteria in Wentworth et al., the antibodies employed in the reference are not specific for the target bacteria (i.e., not capable of binding to the bacteria). Therefore, the rejection of the claimed invention over this reference also cannot be maintained.

From the above, it is clear that the claimed invention is novel over the cited references. Therefore, withdrawal of the present rejections is respectfully requested.

Rejection under 35 U.S.C. §112, 1st paragraph

Claims 4-42, 44, 45 and 54-58 were rejected as allegedly containing new matter. The Examiner asserts that the specification only has support for the recitation of a source of singlet oxygen that is not conjugated to an antibody, but not for the recitation of a source of singlet oxygen that is not covalently attached to the antibody.

As a preliminary matter, Applicants note that independent claim 48 and claims depending therefrom were mistakenly included in the instant rejection. This is because, unlike independent claims 40 and 54, claim 48 does not recite a source of singlet oxygen that is not covalently attached to the antibody.

In addition, Applicants respectfully note that the rational underlying the instant rejection is incorrect. First, the specification provided examples (e.g., Example III and Figures 14A-D) wherein an antibody and a source of singlet oxygen at

different concentrations were mixed for production of reactive oxygen species. These examples inherently and implicitly disclosed that the antibody and the source of singlet oxygen is not covalently attached. There is no covalent bonding between the two molecules to begin with since the two molecules were added as separate molecules at different concentrations. Further, the mere presence of these two molecules in the buffer used in the experiments would not lead to formation of covalent bond between the two molecules. As such, it would be an inescapable conclusion that the antibody and the source of singlet oxygen is not covalently attached to each other in these examples.

Moreover, the plain meaning of conjugation simply means (directly or indirectly) linking two molecules. In the art of antibody conjugates, the conjugation usually involves a covalent bonding between the antibody and the other molecule linked to the antibody. This is evidenced in the attached review article on antibody conjugate technology (Marsh et al., Cancer Treat Res. 1988;37:213-37). Examples of covalent bonding employed in antibody conjugates include peptide bonding formed via a peptide spacer and linkages formed with small organic compound linkers. Assuming that the term "chemical linkages" used by the Examiner in the office action refers to linkages formed by organic compound linkers, such linkages are certainly one type of covalent bonding that is often used in forming antibody conjugates.

Thus, two conjugated molecules in an antibody conjugate may not invariably (although they often do) involve covalent bonding. On the other hand, if two molecules are not conjugated, then there is necessarily no covalent bonding between the two molecules. Therefore, if the specification has taught

embodiments wherein the antibody is not conjugated to the source of single oxygen, the antibody is necessarily not covalently bonded to the source of singlet oxygen.

From the above clarification, it is clear no new matter is introduced because the specification has adequate support for the recitation of a source of singlet oxygen that is not covalently bonded to the antibody. Nonetheless, in a further effort to advance prosecution of the application, Applicants have herein amended claims 40 and 54 by specifying that the source of singlet oxygen that is not conjugated to the antibody. Accordingly, the present rejection should be withdrawn.

Rejection under 35 U.S.C. §112, 2nd paragraph

Claims 40-42, 44, 45 and 48-58 were rejected as allegedly failing to particularly point out and distinctly claim Applicants' invention. The Examiner notes that the recited "microbe" in the claims lack antecedent basis.

In response, Applicants have amended independent claims 40, 48 and 54 by replacing the word "microbe" with "bacterium." Withdrawal of the instant rejection is therefore respectfully requested.

Additional rejection under 35 U.S.C. §102

Claims 48-51 and 53 were rejected as alleged anticipated by Berthiaume et al. (Biotechnol. 12:703-706, 1994). The Examiner alleged that the teaching of antibody-targeted photolysis of bacteria in Berthiaume et al. anticipates the present claims. Applicants respectfully traverse this rejection. This is because claim 48 specifies that "the source of singlet oxygen would not, on its own, inhibit the growth of the bacteria when exposed to light. On the other hand, as acknowledged by the Examiner,

Berthiaume et al. discuss the use of antibody-targeted photolysis of bacteria by using a targeting antibody along with a conjugated photosensitizer which becomes toxic only upon exposure of light. Unlike the presently rejected claims, Berthiaume et al. does not teach or suggest the use of a source of singlet oxygen that could not kill the bacteria even when exposed to light. Accordingly, the instant rejection must be withdrawn.

CONCLUSION

In view of the foregoing, Applicants respectfully submit that the claims now pending in the subject patent application are in condition for allowance, and notification to that effect is earnestly requested. If needed, the Examiner is invited to telephone Applicant's attorney at (858) 784-2937 to facilitate prosecution of this application.

The Director is hereby authorized to charge our Deposit Account No. 19-0962 in the event that there are any additional charges associated with the present Petition or any Response in connection with this application.

Respectfully submitted,

August 28, 2008
Date

Hugh Wang, Reg. No. 47,163

Enclosure: Marsh et al., Cancer Treat Res. 1988;37:213-37.

THE SCRIPPS RESEARCH INSTITUTE
10550 North Torrey Pines Road
Mail Drop TPC 8
La Jolla, California 92037
(858) 784-2937

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13. Antibody-toxin conjugation

Jon W. Marsh, Kasturi Srinivasachar, David M. Neville, Jr.

Introduction

The purpose of this chapter is to discuss our present knowledge concerning the numerous chemical means used to couple toxins to antibodies and to discuss how these methods influence the toxicity of the conjugate. The expanded interest in immunoconjugates is largely the result of two recent developments. The first development is that of monoclonal antibodies [1]. The advantages of monoclonals over serum-derived (polyclonal) antibodies are numerous: 1) specificity in binding to a single (chosen) antigen, 2) singularity in antibody class and thus control over features such as complement fixation, 3) generation of large quantities of the identical antibody and thus reproducibility between different laboratories and different times, and 4) since the immunoglobulin structure is constant, the chemical modification and separation techniques used to conjugate toxin should be both reproducible and more capable of generating a homogeneous product. In addition, the singular specificity and affinity permits meaningful biochemical studies, such as determination of affinity, receptor occupancy, and cellular antigen number, as well as biophysical characterization of the constructed immunotoxins (ITs).

The second development is that of heterobifunctional chemical crosslinkers. The earliest agents used, such as glutaraldehyde [2, 3], toluene diisocyanate [4], and diethyl malonimidate [5], possess homofunctionalities, and thus crosslinked not only the two molecules of interest, but also resulted in undesirable side products such as homopolymers, large protein aggregates, and uncontrolled modification of the antibodies or toxins. The intramolecular crosslinking of toxins can render them nontoxic. The first advance came with the use of chlorambucil derivatives, but yields remained low and undesirable crosslinking persisted [6, 7]. The general procedure for the synthesis of toxin heteroconjugates through the utilization of asymmetric disulfides was set forth by Chang and Neville [8], who 'activated' human placental lactogen with methyl-5-bromovalerimidate. The development of this and other heterobifunctional reagents, such as m-maleimidobenzoyl-N-hydroxysuccinimide ester [MBS; 9, 10], N-succinimidyl 3-(2-pyridyl-dithiolpropionate) [SPDP; 11], methyl 3-mercaptopropionimidate [12], and 2-iminothiolane [12–14]

Frankel, A.E., (ed.), Immunotoxins.

permitted highly specific crosslinking, that is, crosslinking only between antibody and toxin.

Earliest attempts to conjugate toxins and antibody made use of homobifunctional reagents that crosslinked the free amino groups found in abundance on both protein species. The nonspecific crosslinking generated with these reagents was, however, undesirable, and more efficient and specific crosslinking techniques were developed. These generally involve first generating a free thiol group on one protein and, secondly, modification of the second protein to introduce a functionality which will react selectively with those thiol groups. This strategy gained popularity with the realization that thiols are very nucleophilic, can be generated in a controlled manner on one of the two protein moieties, and react rapidly in high yield with suitable acceptors.

The initial modification of a protein, whether the addition of a thiol group or the addition of a moiety which reacts with a thiol, generally involves modification of the amino groups of the protein. Amino groups are reactive, abundant, and, in a limited way for most toxins, expendable. That is, one can modify a limited number of amino groups without diminishing biological activity. The rationale for the introduction of a thiol is two-fold. First, in the normally oxidative environment free thiols are generally absent on the surface of proteins; thus, one can add novel moieties to a selected protein. Secondly, free thiols are reactive. These moieties are readily and specifically modified with reagents such as maleimide and active halides.

The construction of toxin-antibody conjugates has generally involved the placement of a reducible disulfide bond somewhere between the antibody and the enzymatic moiety of the toxin. The synthesis of A chain or A chain-like ribosomal inactivating protein conjugates (described in greater detail in Chapters 7 and 12) has involved the chemical formation of a disulfide bond through the use of reagents such as SPDP. The coupling of an antibody to whole toxin generally involves formation of a thioether between the two macromolecules, but a reducible disulfide still exists between the toxin's A and B chains. With respect to which chain is coupled (via a thioether) to the antibody, whole toxin conjugates are heterogeneous; thus, the preparation is presumably composed of highly active (IgG-s-B-s-s-A) and less active (IgG-s-A-s-s-B) species. The formation of disulfides through the use of reagents such as SPDP is a relatively slow process when compared to thioether formation. Thus, beyond the increase in stability one gets with a thioether, there are very practical reasons, such as product yield, for using thioether linkages in holotoxin conjugate construction.

Chemistry of crosslinking

The coupling of two different protein macromolecules that results in heterodimer formation requires that each protein be modified prior to mixing of

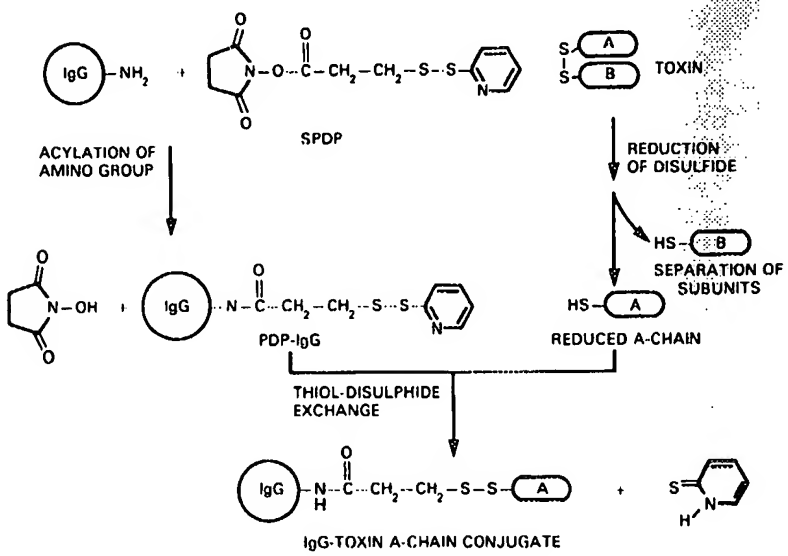


Figure 1. Scheme for synthesis of toxin A chain-immunoglobulin disulfide-linked conjugate. Thiolation of toxin A chain is accomplished by reduction of interchain disulfide; activation of antibody is accomplished with the reagent SPDP, succinimidyl 3-(2-pyridyldithiolpropionate).

the two. These modifications must not result in the formation of homopolymers. The ensuing paragraphs will be concerned with specific details regarding the generation of free sulfhydryl groups on a protein and general methods for 'activating' another protein with a thiol specific moiety.

Generation of thiol groups on first protein

The generation of free sulfhydryl groups has been achieved by two different methods. The first involves reductive cleavage of the native cystine residues in proteins with reagents such as dithiothreitol [DTT; 15] whereas in the second procedure thiol groups are added chemically to the protein (thiolation).

Reductive cleavage of native disulfides. The native disulfides in either the toxin or the antibody can be cleaved to generate free SH groups. An example where an IT is constructed utilizing the reductive cleavage of the A and B chains of the toxin is illustrated in Figure 1.

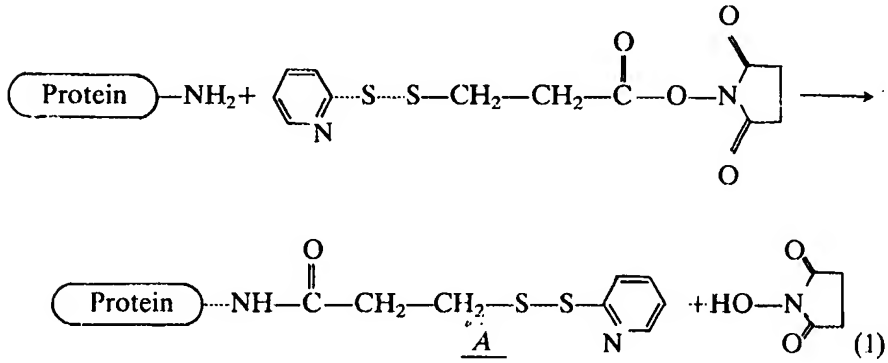
Reductive cleavage of disulfides can also be employed to functionalize antibodies with free thiol groups. This approach is feasible because reduction conditions can be kept mild enough not to alter significantly the functional features of the immunoglobulin [16, 17]. In general, it is necessary to reduce about five cystines (i.e., generate 10 thiol groups) in order to efficiently crosslink the immunoglobulin to an activated toxin [18, 19]. The

concentration of DTT needed varies with the monoclonal antibody, and optimum levels have to be individually determined. Levels too high denature the protein, whereas too low concentrations can lead to inadequate cross-linking. The wide variance in the amount of DTT necessary is clearly demonstrated by a comparison of the monoclonal T101 which requires 10 mM DTT with TA1 and UCHT1 where 100 mM DTT is employed [20, 21].

One can also incorporate a single free thiol group on the $F_{ab'}$ fragment of an antibody. Rabbit IgG upon proteolysis with pepsin yields the disulfide linked fragment $F_{(ab')}$-S-S-$F_{(ab')}$. Selective reduction cleaves this dimeric unit to produce an $F_{ab'}$ moiety in which the light and heavy chains remain associated and the free thiol group is available for coupling [22, 23].

Chemical introduction of thiol groups

The most commonly used reagents for the introduction of free sulfhydryl groups are SPDP and 2-iminothiolane. SPDP reacts with amino groups on proteins as shown below (Equation 1).



(1)

Under carefully controlled conditions the disulfide linkage in A can be selectively cleaved by DTT, generating a thiolated protein without concomitant reduction of native protein disulfide bonds. Carlsson et al. [11], who first introduced the SPDP crosslinker, have shown that at pH 4.5 the reaction of excess DTT with the protein-2-pyridyl disulfide is very specific. This reaction is fairly rapid, even under conditions of low thiolate ion concentration, because of the enhanced electrophilicity of the 2-pyridyl disulfide caused by protonation of the ring nitrogen atom at this pH. In other words, the reaction proceeds in the desired direction because 2-thiopyridine is a good leaving group. These authors have shown the utility of this method using various proteins. When ribonuclease, α-amylase and rabbit anti-(human transferrin) antibodies containing 0.8, 1.0, and 1.7 moles of 2-pyridyl disulfide structures/mole of protein, respectively, were reduced at pH 4.5 with DTT, 0.75, 0.95, and 1.3 moles of thiol group/mole of protein were obtained.

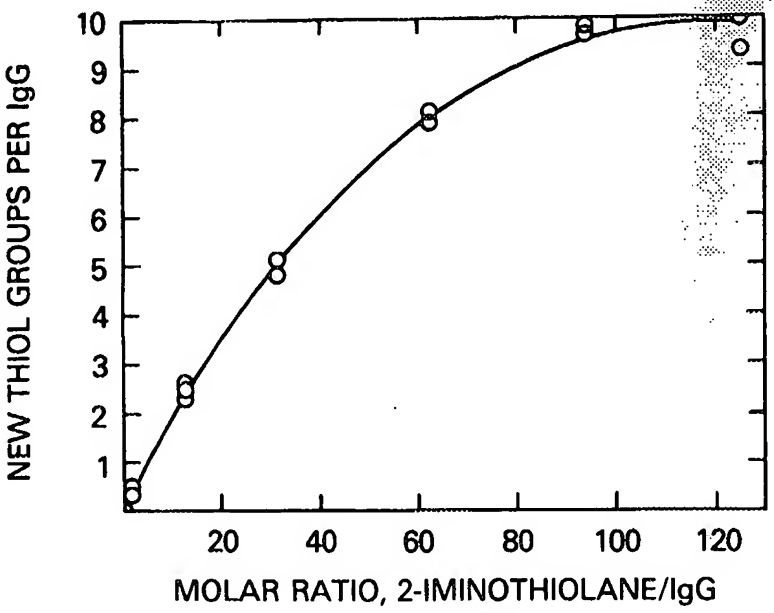
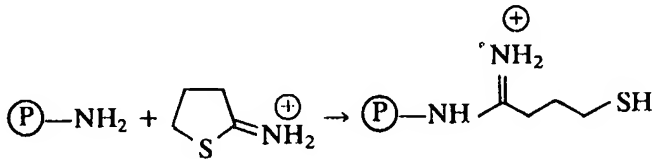


Figure 2. Generation of thiol groups in immunoglobulin with 2-iminothiolane. Varied levels of 2-iminothiolane were incubated with a constant concentration of immunoglobulin (80 μM). Thiol groups were determined with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB).

2-iminothiolane also adds to amino groups on proteins to generate a free sulfhydryl group via a ring opening reaction (Equation 2).



(2)

The introduction of new thiol groups on proteins can be followed by incubating a constant concentration of protein with varying levels of 2-iminothiolane. An example where this was carried out on immunoglobulin is shown in Figure 2 [24]. The thiol groups were determined using 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB). It was found that only one or two iminothiolane generated thiol groups were necessary for coupling the antibody to activated ricin. This is in contrast to procedures in which free sulfhydryl groups were generated on the antibody by reducing cystine residues, where it was determined that optimally 10 thiols groups per immunoglobulin had to be introduced for antibody-ricin conjugation. The thiols generated by 2-iminothiolane are presumably all reactive, whereas in the other procedures some of the cystine residues are presumably not sterically accessible to the

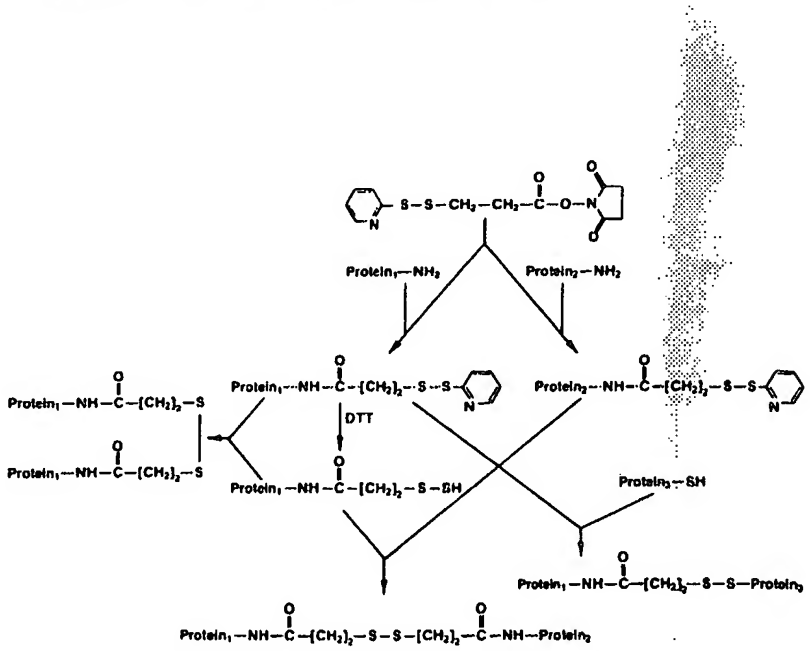


Figure 3. Generation of disulfide linked proteins with SPDP (from reference 1).

activated second protein. 2-iminothiolane has the further advantage that it can modify proteins with conservation of the positive charge. Consequently, there is little or no effect on the conformation of the protein, and the biological activity is usually retained [25, 26].

Generation of a thiol specific functionality on the second protein

Once the first protein has been thiolated, the second protein has to be activated, that is, modified in such a way that it will react specifically with the thiol group on the former. Depending on the nature of the modification, the two proteins can be coupled either via a cleavable disulfide bond or a stable thioether linkage.

Activation resulting in disulfide coupling. A disulfide linkage results when the thiol group on the first protein reacts with a disulfide on the second protein with the formation of a new disulfide bond (Equation 3).

$$\text{(P}_1\text{)—SH} + \text{(P}_2\text{)—S—S—R} \rightarrow \text{(P}_1\text{)—S—S—(P}_2\text{)} + \text{RSH} \qquad (3)$$

For this reaction to proceed in the desired direction, RSH must be a good leaving group. The heterobifunctional reagent, SPDP, which has an excellent leaving group in the thiopyridyl moiety, is commonly used for this modification (Figure 3). Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid) too can be employed in forming disulfide linked conjugates, since 5-thio-2-nitrobenzoic acid is also a very good leaving group. Raso and Griffin [27] have demonstrated the utility of Ellman's reagent in their preparation of a human immunoglobulin directed $F_{ab'}$-ricin A chain conjugate.

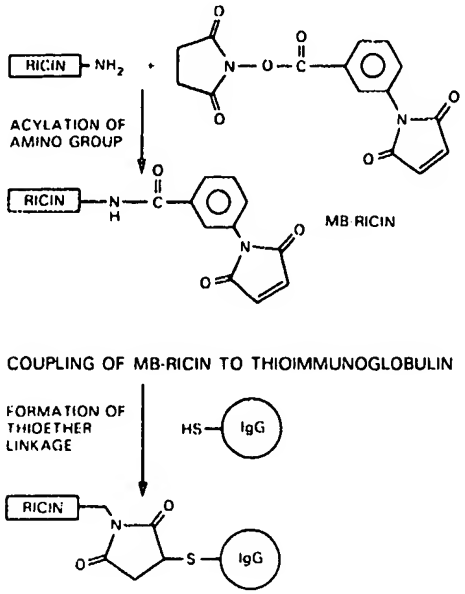


Figure 4. Scheme for synthesis of whole toxin-immunoglobulin conjugate with a thioether linkage. Thiolation of IgG can be accomplished by either the chemical addition with 2-iminothiolane or by limited reduction with dithiothreitol (DTT). Activation of the toxin ricin is accomplished with m-maleimidobenzoyl-N-hyroxysuccinimide ester (MBS).

Activation leading to thioether formation. This sort of activation has been mainly achieved through the maleimide functionality, which is highly specific for thiol groups. Many different crosslinkers which incorporate the maleimide moiety are known, and one representative example is MBS. Its use in the synthesis of an ITn conjugate is schematically illustrated in Figure 4. In our laboratory we have employed MBS successfully in ITn preparation and have optimized conditions for effective crosslinking with this reagent. MBS was incubated with ricin at various molar ratios, and then thiols in the form of radiolabeled cysteine were added [24]. This study showed that one must add approximately five moles of MBS to one mole of ricin to couple one mole of cysteine (Figure 5).

Since effective use of these crosslinkers depends on an intimate knowledge of the reactions and stability of maleimides, a fairly detailed discussion of the chemistry of this functional group follows.

Chemistry of maleimides

Much of our knowledge concerning the reactivity and chemistry of maleimides derives from data on N-ethylmaleimide, the most extensively studied derivative. Thiols, which are excellent 'soft' nucleophiles, undergo a Michael

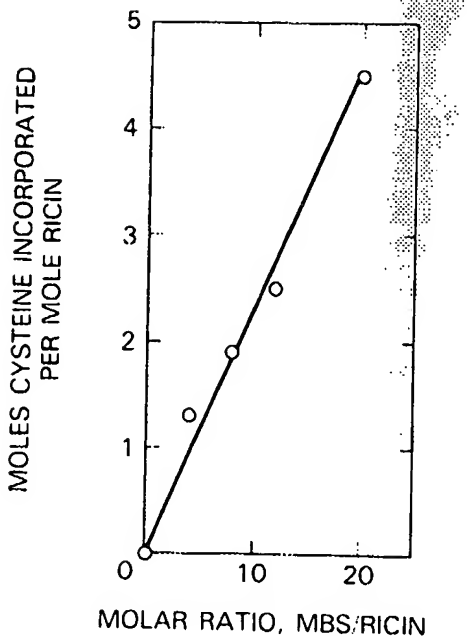
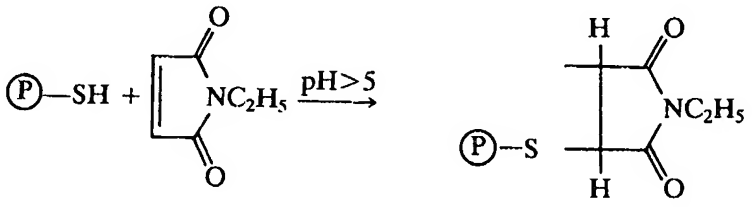


Figure 5. Addition of cysteine residues to MBS activated ricin. After incubation with MBS, ricin was exposed to an excess of ^{14}C-cysteine, and the incorporation of label was determined. The molar ratio is a measure of MBS input to a constant (10 mg/ml) ricin concentration.

reaction with maleimides to yield exclusively the adduct to the double bond (Equation 4).



(4)

The resulting thioether bond is very stable and cannot be cleaved under physiological conditions. The addition of the sulfhydryl component to the double bond of maleimides has been convincingly demonstrated by the isolation and characterization of the products from the reaction of L-cysteine and glutathione with N-ethylmaleimide [28]. In both cases the adducts (B or C) were obtained in nearly quantitative yield.

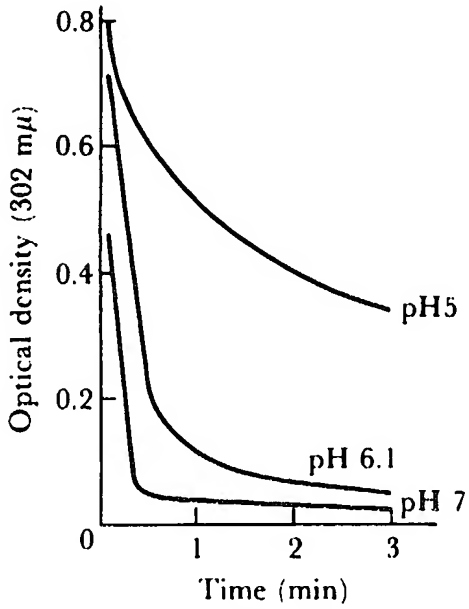
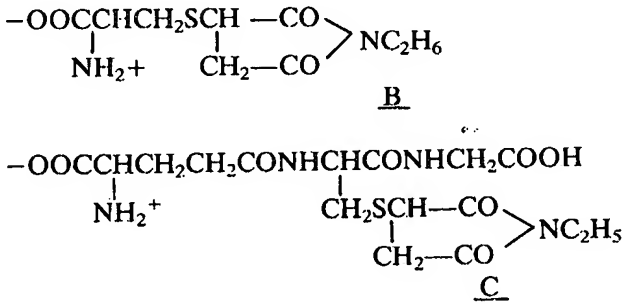


Figure 6. Variation of the rate of reaction of NEM and glutathione (GSH) with pH. Both reactants are 10^{-3} M in 0.1 M sodium acetate, pH 5.0; sodium phosphate, pH 6.1 and pH 7.0 (from reference 29).



B

C

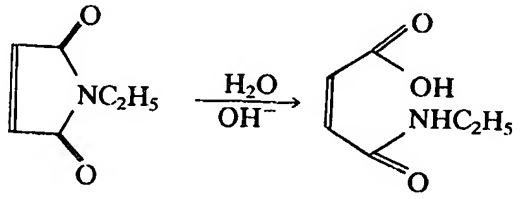
The quantitative reaction of maleimides with thiol groups forms the basis for a spectrophotometric assay of the latter. At pH 6.0, the loss in absorption of N-ethylmaleimide at 300 nm can be used for the quantitative estimation of sulfhydryl groups in dilute solution in the presence of excess N-ethylmaleimide. However, most laboratories prefer to use Ellman's reagent to estimate the degree of thiolation of a protein because of its greater sensitivity.

Gregory [29] did some preliminary measurements of the rate of the reaction of N-ethylmaleimide with glutathione as a function of pH and found that the reaction was markedly accelerated in going from pH 5 to pH 7 (Figure 6). Gorin et al. [30] studied the kinetics of the reaction between N-

ethylmaleimide and cysteine in greater detail. The reaction was found to be second order with a specific rate constant at 25°C and pH 4.95 of 14 liter $mole^{-1}$ sec^{-1}. It was estimated that at pH 7, the rate would be 1.53×10^3 liter $mole^{-1}$ sec^{-1}, implying that in a reaction mixture 10^{-3} M in N-ethylmaleimide and mercaptan, the half-life period would be only 0.7 seconds. From this data it is clear that the optimum pH for linkage of thiol containing proteins to maleimide derivatives is between 5 and 7. Although the rate of the reaction between the sulfhydryl group and maleimide increases sharply at alkaline pH (because of the enhanced nucleophilicity of mercaptide ion RS^- over RSH), competing reactions become significant under these conditions.

Two major side rections are of concern:

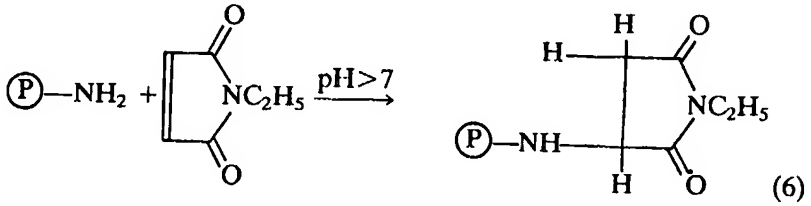
1) The decomposition of the maleimide reagent by hydrolysis to maleamic acid (Equation 5). The rate of decomposition only becomes significant at pH above neutrality [29]. At pH 7 the hydrolysis has an apparent rate of 3.2×10^{-4} min^{-1} in 0.1 M sodium pyrophosphate buffer at 20°C [31] — too slow to interfere with the reaction of N-ethylmaleimide with sulfhydryl containing proteins. Furthermore, the product of the hydrolysis, maleamic acid, reacts only very sluggishly with mercaptans.



(5)

2) Reaction with free amino groups. Amino groups can conceivably react with maleimides in either of two ways:

a) add to the double bond of the maleimide ring (Michael reaction analogous to the addition of SH) (Equation 6).



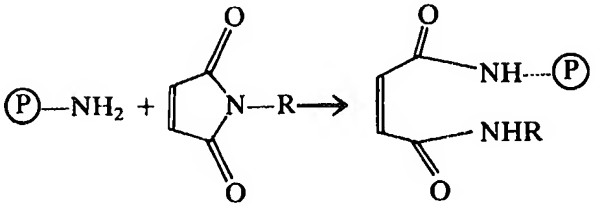
(6)

or b) undergo acylation by addition to the carbonyl group followed by ring opening (Equation 7).

Table 1. Half-lives for the reaction of peptides with N-ethylmaleimide [ref. 33]

Peptide[1]	$t^{1/2}$ (hr)
Glycyl-alanine	1.2[2]
Peptide Tα-4[1] (Val-Leu-Ser-Pro-Ala-Asp-Lys)	3.2[3]
Peptide Tβ-6[2] (Val-His-Leu-Thr-Pro-Glu-Glu-Lys)	2.5[3]

[1] 1 mM peptide and 10 mM N-ethylmaleimide in all cases
[2] 37°C
[3] 25°C



(7)

There are conflicting reports in the literature as to which of these two pathways is followed in the reaction of the amino groups of peptides with N-ethylmaleimide. Smyth et al. [32] reported that the α-amino groups of peptides and amino acid derivatives reacted with N-ethylmaleimide at pH 7.4 to give acylated products of the type RNH-CO-CH = CH-CONHR'. However, no products were isolated in pure form from these preparations. Smyth et al. have later re-examined the reaction of N-ethylmaleimide with peptides and amino acids [33]. This time they carried out a preparative scale reaction between glycylalanine and N-ethylmaleimide and isolated the product by chromatography as an oil which failed to crystallize. On the basis of indirect evidence, these authors propose that the α-amino groups of the peptide add to the double bond of the maleimide ring in a completely analogous fashion to the reaction of sulfhydryl groups with N-ethylmaleimide. This seems reasonable in view of the fact that maleimides are not very effective acylating agents and no other additions to the carbonyl group besides hydroxide ion are known to occur under mild conditions. The same publication also provides data on the rates of reaction of three peptides with N-ethylmaleimide at pH 7.4 (0.2 M sodium phosphate buffer) (Table 1).

In the absence of any definitive work on the reaction of amino groups with maleimides, the exact structures of the products are still open to debate. It could well be that addition occurs both to the double bond as well as the carbonyl groups of the maleimide to give a mixture of products. Regardless

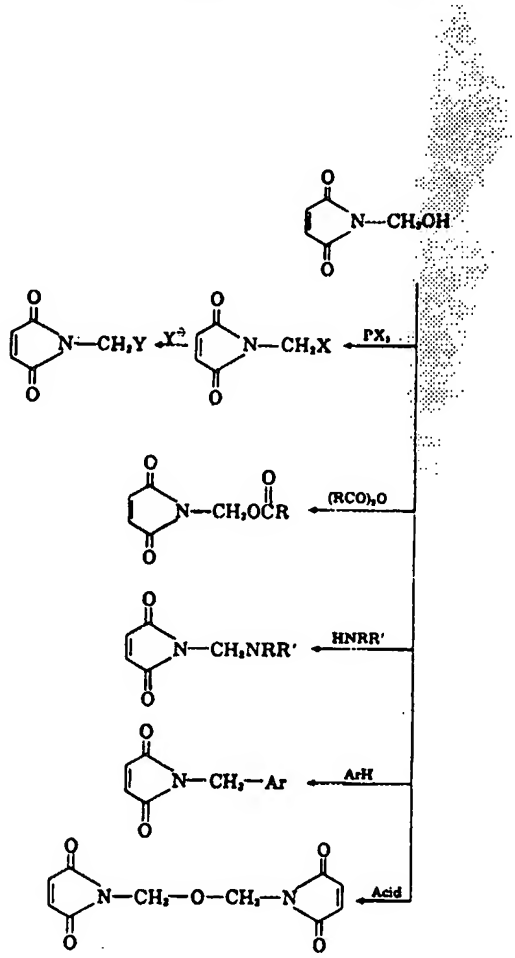
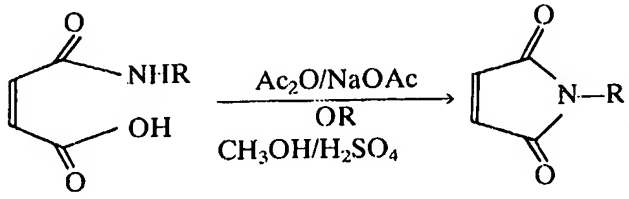


Figure 7. Formation and reactions of some maleimide derivatives (from reference 34).

of the identity of the products, it should be emphasized that amino groups can effectively compete with thiols for addition to maleimides at pHs above neutrality.

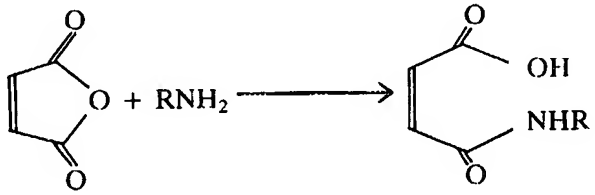
Maleimides in crosslinkers

Besides being thiol specific, maleimides can be relatively easily derivatized, and this is of advantage in the construction of heterobifunctional crosslinkers. Since the double bond of the maleimide needs to be left intact for addition to thiol groups, most derivatives are formed by reaction at the nitrogen atom. Some representatives derivatives and their synthesis [34] are outlined in Figure 7. Care should be exercised when making these derivatives to avoid basic conditions, since, in addition to the aforementioned hydrolysis in aqueous solution, maleimides readily undergo base catalyzed polymerization even in nonaqueous media. Another useful reaction in the synthesis of maleimide derivatives is the facile dehydrative cyclization of maleamic acids to maleimides [35] (Equation 8).



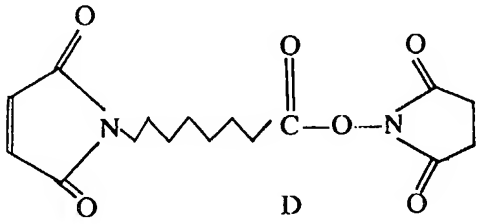
(8)

The maleamic acids themselves are formed in good yield upon the addition of the corresponding amines to maleic anhydride [35] (Equation 9).



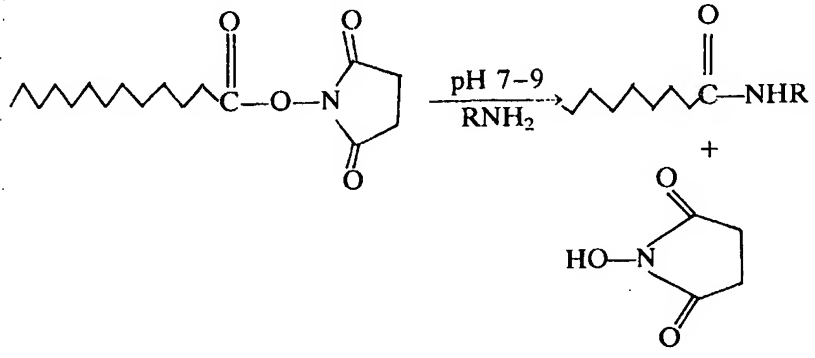
(9)

Many commercially available heterobifunctional crosslinkers contain a N-hydroxy succinimidyl ester in conjugation with a maleimide moiety (see structure D). MBS and other variants are examples of this type of non-reversible crosslinkers.



D

N-hydroxy succinimidyl esters are popular because they are highly activated and react with amino groups under mild conditions (pH 7–9). An advantage of this functionality over the older hydroxyphthalimide esters is the water solubility of the product of the reaction with amino groups, namely N-hydroxysuccinimide [36] (Equation 10).



(10)

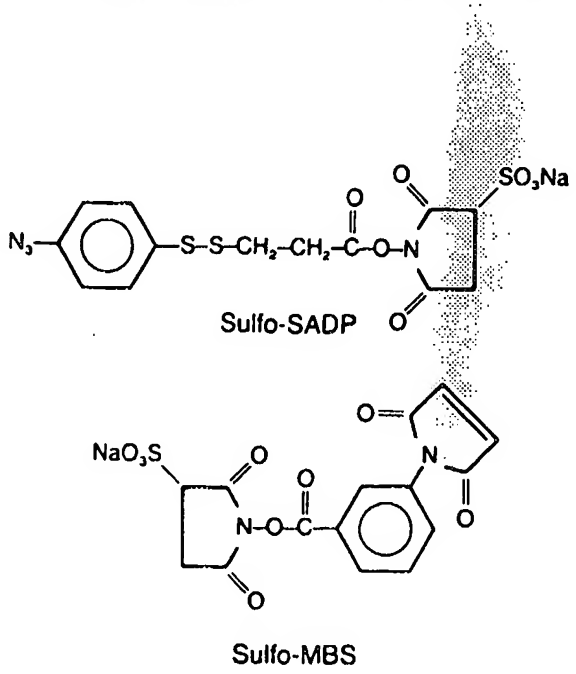


Figure 8. Some examples of water soluble heterobifunctional crosslinkers.

The addition of N-succinimidyl reagents to the amino groups of proteins results in the formation of an amide linkage, identical to that found in peptide bonds, which, although possibly susceptible to enzymatic cleavage (see Chapter 14), is quite stable to the chemical extremes seen in biological systems. Unlike the amidine bond formed through the interaction of imidates or 2-iminothiolane with amino groups, the amino group modified with a N-succinimidyl group loses its positive charge.

Novel crosslinking reagents

Water soluble crosslinkers

Most of the commercial bifunctional crosslinkers are fairly nonpolar organic molecules which are insoluble in aqueous media. For efficient crosslinking, these have to be dissolved in water-miscible organic solvents such as dimethyl formamide (DMF), acetonitrile, or tetrahydrofuran (THF), which can on occasion have undesirable effects on proteins. To circumvent this, many water soluble versions of the older crosslinkers have recently been introduced and some examples are shown in Figure 8 [37]. In all cases, water solubility is conferred by the presence of a sulfonate group, e.g., $-SO_3Na$. These reagents were in general designed for the crosslinking of cellular surface proteins in situ because their hydrophilicity prevents intracellular access. In our opinion, such reagents offer only a slight advantage over the traditional water insoluble ones for toxin-antibody conjugation. If only the minimum volume of organic solvent is employed to dissolve the reagents, most

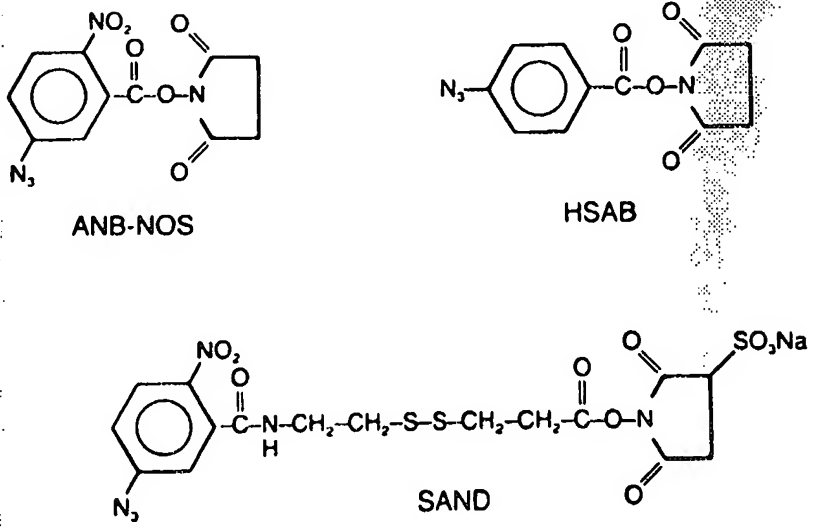
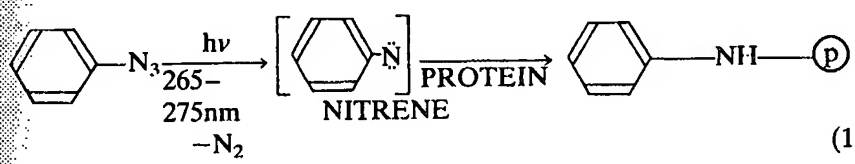


Figure 9. Representative examples of crosslinkers which contain photoreactive groups.

problems like the precipitation of the proteins can be avoided. It is also important to try out different organic solvents in order to optimize crosslinking efficiency.

Photoreactive crosslinkers

Bifunctional crosslinkers containing a photoreactive group are also commercially available. Most of these are based on phenyl azide or nitrophenyl azide moieties which on irradiation generate highly reactive nitrenes (Equation 11).



(11)

The introduction of a nitro group into the aromatic ring allows for initation of photoactivation at 320 nm–350 nm which minimizes the undesirable side reactions likely to occur at lower wavelengths. Some examples of these crosslinkers are shown in Figure 9.

Although these reagents have been used to crosslink proteins to membrane components with some success [38, 39, 40, 41], they have serious drawbacks. The extremely high reactivity of the nitrenes generated upon irradiation allows them to bind covalently to almost any macromolecular component, resulting in nonspecific crosslinking. Furthermore, nitrenes also

react with water and buffer components and as a consequence yields of covalent products are often low. For these reasons azide based crosslinkers will be of very limited utility in IT synthesis.

Cleavable crosslinkers

Crosslinking reagents containing a disulfide bond [42, 43, 44] or a vicinal glycol unit [45, 46] are well known and have been extensively used to gain information about the arrangements of proteins in ribosomes, membranes, etc. In the IT field, other than disulfides, cleavable crosslinkers have not been much utilized to date, and there are no reports in the literature regarding the biological evaluation of conjugates with a nondisulfide labile bond. For example, the vicinal glycol linkage can only be cleaved under rather harsh conditions, e.g., periodate oxidation, which can damage proteins.

A new and more promising cleavable crosslinker has been recently reported [47, 48]. This reagent employs an acid labile linkage to crosslink two different proteins. Above pH 7 the linkage is stable, but on mild acidification (pH 4–5) cleavage occurs to yield monomeric proteins. An interesting feature of this reagent is that one of the proteins is released in native form, which could be an asset in the synthesis of ITn conjugates. The acid lability of this reagent is based on the well known citraconic anhydride system. The reported synthesis of this crosslinker E involves a fairly long sequence of steps. Its use has been demonstrated by the construction of a conjugate F between monoclonal antibody J5 and gelonin (Figure 10). The formation of this conjugate resulted in almost total loss of the ribosome inactivating ability of gelonin. However, on acid catalyzed hydrolysis of the amide bond linking gelonin to the rest of the conjugate, native gelonin was released resulting in complete restoration of its toxic activity (Figure 11).

A different type of crosslinking reagent G, which can be cleaved with low-energy UV light (365 nm), has recently been reported [49]. ITs H of pokeweed antiviral proteins (PAP-S) and the murine monoclonal antibody J5 were prepared and shown to release monomeric PAP-S upon irradiation (Figure 12). The authors claim that this new method of reversible protein crosslinking may prove useful for the release of toxins or drugs at sites accessible to light.

Effects of chemical modification on biological activity

To achieve the highest yield of crosslinking between toxin and antibody, one might wish to generate high numbers of thiols on one species and multiple activation moieties (e.g., maleimide groups) on the second protein. This is not done for two reasons. The first is that the desired product, a biologically efficacious conjugate, is found at relatively low toxin to antibody ratios [see

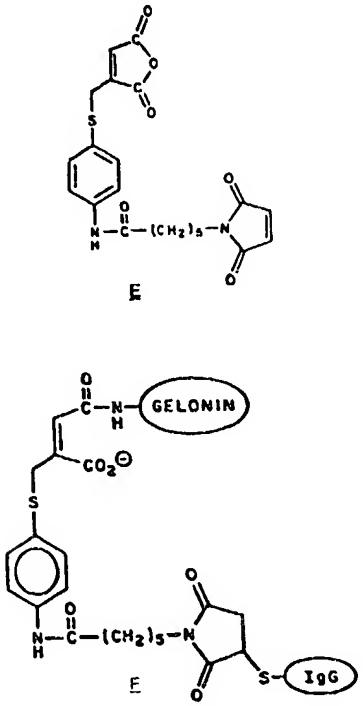


Figure 10. An example of an IT F formed by conjugation of the toxin, gelonin, with an antibody using an acid cleavage crosslinker E.

below and ref. 19]. Secondly, increased chemical modification of either the toxin or antibody generally results in decreased bioactivity.

As previously mentioned, antibodies vary in their sensitivity to reduction with DTT, which is used to generate free thiol groups. DTT levels which are too high denature the protein, and levels which are too low result in an inadequate number of thiol groups and, consequently, inadequate crosslinking. For example, T101 is normally reduced with 10 mM DTT, whereas TA1 and UCHT1 are incubated with 100 mM DTT [20]. Obviously, the sensitivity of the antibody to various chemical modifications may vary from antibody to antibody.

In addition, the coupling of a toxin to an antibody molecule may sterically hinder the binding sites of either moiety. For example, the conjugation of ricin to the antibody, OX-7, resulted in loss of half the antibody binding capacity [19].

The effects of chemical modification of toxins such as ricin have been more extensively studied. The reagents used to couple ricin to antibodies generally have involved modification of the toxin's amino groups. It has previously been shown that modification of six amino groups with the reagent, trinitrobenzenesulfonate resulted in an 88% decrease in the LD_{50}

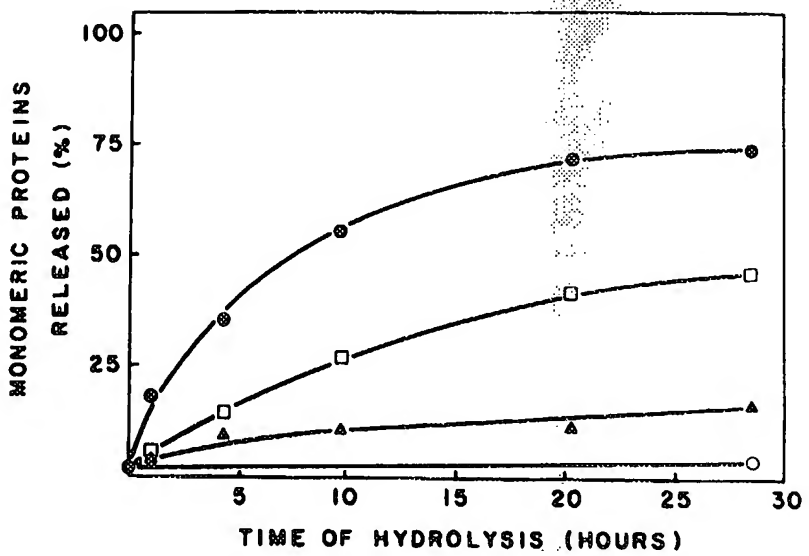
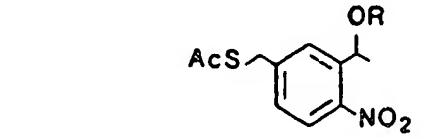


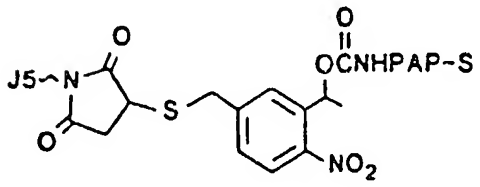
Figure 11. Acid catalyzed release of monomeric proteins from complex F estimated by quantitative scanning of polyacrylamide sodium dodecyl sulfate gels. The ordinate shows the percent of monomeric proteins released (sum of stain associated with all peaks), and the abscissa shows the time of hydrolysis at 37°C in citrate phosphate buffers at pH 4.0 (closed circles), pH 5.0 (open squares), pH 6.0 (closed triangles), and pH 7.8 (open circles) (from reference 48).

values of ricin in mice [50]. However, this is in contrast to the lack of effect on in vitro potency of ricin which has been succinylated [six amino groups, ref. 51]. Other types of modifications, such as that of the tryptophan residues with N-bromosuccinimide [2 to 6 residues; refs. [50, 51] resulted in a greater than 90% loss of in vitro activity. O-acetylation of one or two tyrosines with N-acetylimidazole resulted in a 90% loss of cellular toxicity [52]; the tyrosines were shown to be integral in the B subunit binding site. Ricin is known to be a glycoprotein with high mannose-type carbohydrates on both subunits [53]. One can specifically generate carbonyl groups on the terminal mannosyl residues through periodate oxidation, and these groups could be available for crosslinking to a well defined area on the ricin molecule. However, considerable evidence exists that this modification, even when immediately followed by reduction of the carbonyl groups, reduces biological activity. Oxidative destruction of 13 to 18 mannose residues resulted in a 90% or greater loss in cellular toxicity, although hemagglutinating activity, a B chain function, and inhibition of protein synthesis in a cell-free system, an A chain function, were fully retained [54, 55]. The 37°C binding affinity of the modified and the native toxins remained identical [55]. Reconstitution of modified ricin A chain with native B chain resulted in a toxin equally potent to that of native ricin, suggesting that the alteration occurred in the B subunit. The importance of the B chain has previously been demonstrated from other work and is discussed later in this chapter.



R=COCl

G



H

Figure 12. The use of crosslinking reagent G in forming a photocleavable link between PAP-S and J5 antibody.

Steric considerations

The mere act of crosslinking antibodies to toxins will sterically hinder the biochemical functions of both moieties. This may explain why no known antibody conjugated to ricin or diphtheria toxin possesses the efficacy of the native toxin on sensitive cells. The coupling of ricin to an antibody, through a thioether linkage formed by thiolating the antibody with 2-iminothiolane, resulted in a conjugate with less than one half the antibody binding capacity of the unmodified antibody [19].

The number of toxin molecules per antibody also affects the overall efficacy of the conjugate. The addition of a second toxin molecule to a diphtheria toxin-polyclonal anti-(human lymphocyte) immunoglobulin resulted in a 75% loss in toxicity [7]. The addition of a third caused greater losses. With a monoclonal antibody DT conjugate, we have found that the monotoxin conjugate is nearly three-fold more potent and a third more efficacious than the bitoxin conjugate [56]. The results have been different for ricin-antibody conjugates (see Figure 13). Here, the addition of second ricin (IgG-R_2) resulted in an eight-fold increase in potency, and the rate of protein synthesis inhibition increases from slightly more than 0.1 log/hour (for the IgG-R_1 species) to 0.4 log/hour [both conjugates at 200 ng/ml; ref. 19]. The addition of third ricin (IgG-R_3) resulted in a drop of the rate to 0.2

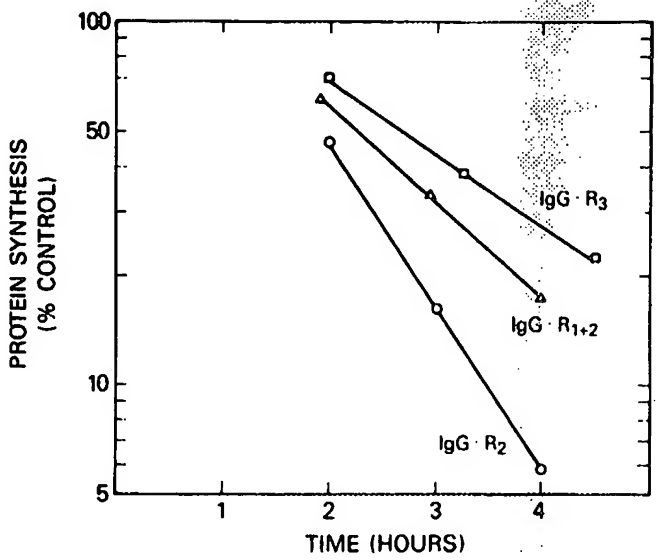


Figure 13. Kinetics of protein synthesis inhibition by antibody-ricin conjugates. Three different conjugates possessing the same antibody, but varied levels of ricin (R_1, R_2, R_3, representing one, two, and three ricins per IgG, respectively), were assayed for their ability to inhibit cell protein synthesis (from reference 19).

log/hour. Although steric hindrance may play in role in the overall toxicity and may be responsible for the decrease seen with the triricin species, other factors play a role in the efficacy of ricin conjugates. Even with the possibility of increased steric hindrance, maximal activity is achieved with more than one ricin molecule per antibody.

Peptide spacer conjugates

Several lines of evidence indicate that the efficacy of whole toxin conjugates is dependent on the toxin moiety's B subunit interaction with a vesicular/organelle membrane. First was the demonstration that whole ricin conjugates were more efficacious than A chain conjugates and that the addition of free ricin B chain to cellular bound ricin A chain conjugates increased the rate of protein synthesis inhibition five-fold [58]. This idea was further expanded with the demonstration that the toxicity of ricin A chain conjugates was enhanced by the addition of B chain conjugates [59]. These results implied that the B chain directly affected efficacy, and that a ricin-antibody conjugate, whose B chain-membrane interactions were optimized, would have higher efficiency. Such a conjugate was constructed as outlined in Figure 14. This involved the coupling of the toxin to the antibody through a 29 residue polypeptide derived from thiolation of the oxidized insulin B

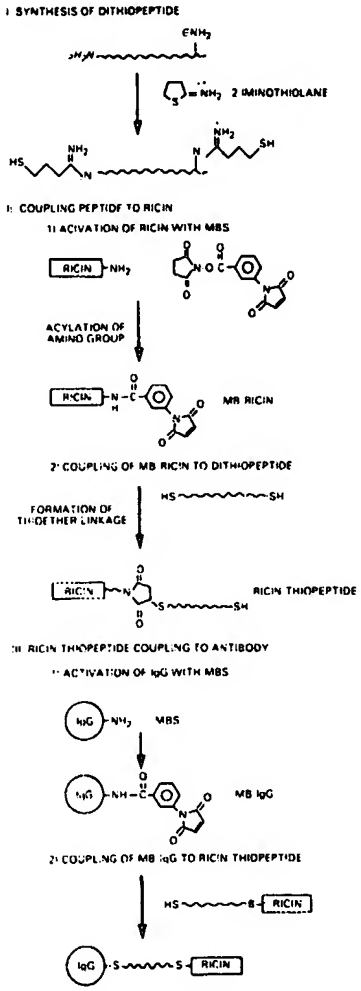


Figure 14. Scheme for synthesis of peptide-linker conjugate. Thiolation of the two amino groups of oxidized insulin B chain by 2-iminothiolane is followed by addition of MBS-treated ricin to one end and MBS-activated IgG to the other (from reference 24).

chain [57]. When compared to a more conventionally constructed conjugate, the peptide spacer IT was found to possess a 10-fold increase in potency and a two-fold increase in efficacy. We have suggested that these effects are due to minimized steric constraints on both the toxin and antibody moieties.

Conclusion

When synthesizing a new IT, one must determine the effects that the conjugation will have on both antibody and toxin functions. How does thiolation or

activation affect antibody binding or the toxin functions of binding and intoxication? How does the coupling of the two macromolecules affect the same functions? Thus, the specific reagents used, as well as the stoichiometry of toxin and antibody molecules, will affect the final efficacy and potency. The methods used to determine these values are discussed in Chapter 13.

The enhancement seen with the peptide spacer is but one indication that ITs are yet to be fully optimized; thus, no general ideal crosslinker exists today. We would speculate that those holotoxin conjugates that will permit the toxin moieties to operate intracellularly, as the native toxins do, will have the highest efficacy possible. Crosslinkers that release (intracellularly) the toxin moiety intact would then be the most efficacious.

If an antibody-toxin conjugate is expected to be of value in the clinics, then an evaluation of that IT with respect to potency and efficacy should be performed. The potency of a conjugate closely correlates with its binding affinity; the data generated on dose-response curves describes only the first log (or 90%) of inhibition. From such a curve, there is no way to extrapolate to multiple log kills. Efficacy, a measure of how many cells a reagent will kill, correlates with the rate-limiting step in the intoxication process. While the rate of intoxication is dependent on receptor occupancy, it is not a linear function [19], and in present clinical application, such as allogeneic and autologous bone marrow transplantation [60], antigen saturation by the antibody moiety is approachable. Under these conditions, the maximal target cell kill for a conjugate, the classical definition for efficacy of such an agent, can be realized. Maximal inhibition rates are achieved for both A chain [58] and whole toxin [19] conjugates under in vitro conditions and, at least for the A chain conjugates, the inhibition rate saturation can be correlated directly with antibody saturation of the cellular antigen.

Presently, ITs are limited to in vitro clinical utility. Whole ricin conjugates require the addition of lactose to prevent nonspecific intoxication, and A chain conjugates require ammonia or monensin to achieve a useful level of cell kill [18, 61]. The solution to the in vivo problem may involve the development of novel chemical crosslinkers which result in the reversible blockade of the whole toxin binding site [60]. Additional solutions will come when we understand the mechanisms of native toxin entry and can use these processes in an opportunistic fashion in our design of new ITs.

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